AD_____

Award Number: DAMD17-99-1-9090

TITLE: Isolation of Signaling Molecules Involved in Anigiogenic Pathways Mediated Alpha V Integrins

PRINCIPAL INVESTIGATOR: Marina Cardo-Vila

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (15 Jul 99 - 15 Jul 00)

4. TITLE AND SUBTITLE

Isolation of Signaling Molecules Involved in Angiogenic Pathways Mediated by Alpha v Integrins

5. FUNDING NUMBERS

DAMD17-99-1-9090

6. AUTHOR(S)

Marina Cardo-Vila

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Burnham Institute

La Jolla, CA 92037

E-Mail: mcardovi@mdanderson.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Integrins that bind to vitronectin are highly expressed in neovasculature and play an important regulatory role in angiogenesis. There are at least two cytokine-dependent pathways that lead to angiogenesis in vivo, and there is evidence indicating that they can be distinguished by their dependency on specific αv integrins. Here we aim to define what molecules are involved in αvβ3- and αvβ5- selective angiogenic signaling. We hypothesize that: (I) molecules that associate with either of those integrins after angiogenesis is triggered by defined cytokines are different; (ii) the assembly of specific molecules associating with the β3 or β5 cytoplasmic domains results in selective signaling. The strategy that will be used to approach these questions is based on the panning of phage peptide libraries of β3 and β5 cytoplasmic domains. We will also investigate whether phosphorylation events can modulate the interaction with such integrin cytoplasmic domain-binding peptides. Finally, by using microinjection-based techniques, we will study the effect of integrin cytoplasmic domain binding peptides in cell adhesion, migration and proliferation upon stimulation with factors that activate endothelial cells in vitro. These studies will shed light into molecular basis of selective signal transduction pathways initiated by αvβ3 and αvβ5. New ways of inhibiting angiogenesis, and ultimately, new strategies to treat breast cancer may result from this work.

14. SUBJECT TERMS

Angiogenesis, integrins, signal transduction

15. NUMBER OF PAGES

19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is a requirement for malignant tumor growth and metastasis (1-3). In the absence of angiogenesis, local tumor expansion is suppressed at a few millimeters and cells lack routes for distant hematogenous spread. Clinical studies have demonstrated that the degree of angiogenesis is correlated with the malignant potential of several cancers, including breast cancer and malignant melanoma (4-7).

Integrin function is regulated by cytokines and other soluble factors in a variety of biological systems. Most commonly, exposure to such factors leads to conformational alterations that result in changes in the activation state of the receptors. Changes in integrin-dependent adhesion ultimately activates various complex signal transduction pathways. Cytoplasmic domains are key regulators of integrin function (8-14). Although the cytoplasmic domains of the various ß subunits share similar primary structures, they differ in certain functional characteristics, since they are responsible for the regulation of receptor distribution and recruitment to the focal adhesion sites (15,16). Thus, ß chain cytoplasmic domains are critical for integrin-mediated signaling into the cell (outside-in signaling) and activation of integrin-ligand binding activity (inside-out signaling) (9, 17-19).

Several lines of evidence have implicated the integrins αvß3 and αvß5 in the angiogenic process. It has been shown that αv integrins are selectively expressed in angiogenic vasculature but not in normal vasculature (20,21). Moreover, αv integrin antagonists can block the growth of neovessels through induction of endothelial cell apoptosis (22-26). Concordant with these findings, it appears that two distinct cytokine-dependent pathways that lead to angiogenesis depend on specific αv integrins. Angiogenesis initiated by bFGF can be inhibited by an anti-αvß3 blocking antibody, whereas VEGF-mediated angiogenesis can be prevented by a blocking antibody against αvß5.

Although both αv integrins bind to vitronectin, they are likely to mediate different post-ligand binding events. For instance, the integrin αvß5 fails to promote cell adhesion, spreading, migration and angiogenesis in the absence of exogenous soluble factors. On the other hand, the αvß3 can induce such events without additional stimulation by cytokines (27-29). Experiments designed to study the molecular basis for cytokine regulation of αvß5 function have shown that upon binding to immobilized vitronectin, inactivated αvß5 is barely detectable in association with actin, α-actinin, talin, tensin, $p130^{cas}$ and vinculin. In contrast, αvß3 induces the localized accumulation of such molecules. Upon activation of PKC, αvß5 can then behave similarly to αvß3, but can not recruit talin (28). Moreover, calphostin C, an inhibitor of PKC, appears to block angiogenesis mediated by αvß5, but not αvß3 (29). These observations suggest that PKC activation probably affects the conformation and/or phosphorylation state of the ß5 cytoplasmic domain. Therefore, cellular events mediated by αvß3 or αvß5 are clearly controlled by different mechanisms (reviewed in 30-33).

Little is known about molecules associated to the cytoplasmic domains of β3 or β5 chain. Two-hybrid screenings with the ß3 cytoplasmic domain provided evidence for a specific interaction between the β3 cytoplasmic domain and ß3-endonexin (34,35). A few other proteins have been reported to interact with ß integrin cytoplasmic domains in general: talin (36), filamin (37), α-actinin (38,39), focal adhesion kinase (40) and the serine/threonine kinase ILK (41), and skelemin (42). Many studies suggest that the molecules which associate with either ß3 or ß5 after the angiogenesis switch is triggered are different. We

hypothesize that the assembly of specific molecules associating with the respective cytoplasmic domains may explain their selective signaling properties.

PROPOSAL BODY

The tasks approved for this proposal are listed below:

Task 1. To select peptides that bind specifically to ß3 or to ß5 integrin cytoplasmic domains. (Months 1-12)

- Construction of phage display peptide libraries and panning on ß3 and ß5 cytoplasmic domain fusion proteins.
- Characterization of ß3 and ß5 cytoplasmic domain-binding phage

Task 2. To investigate whether phosphorylation events can modulate the interaction of the selected peptides with integrin cytoplasmic domains. (Months 13-24)

- Biopanning using phosphorylated libraries

- Binding assays to investigate the affinity of novel phosphorylated peptides

- Investigate if the ß3 binding peptides already isolated may be specifically phosphorylated *in vitro* by known protein-kinases involved in classical signal transduction pathways.

- Evaluate the specificity and affinity of the phosphorylated peptides

Task 3. To determine the biological properties of the cytoplasmic domain-binding peptides. (Months 25-36)

- Microinjection of cells with integrins ß3 and ß5 cytoplasmic domain-binding peptides after stimulation by selected angiogenic factors.

- Cell adhesion, migration and proliferation studies

In the following pages, we report in significant progress on each specific aim during the past year.

We offer experimental evidence to support the rationale and feasibility of this proposal. We introduce the peptide sequences that have been isolated after panning of phage libraries with the cytoplasmic domain of ß3 and ß5.

First, we show data related to the specificity and binding properties of the peptides, including the ones isolated from Tyr-containing peptide libraries. Second, we show results from homology searches using the most promising peptides, and describe the development and testing of anti-peptide antibodies.

Third, we demonstrate that the penetratin peptide chimeras are internalized by tumor and endothelial cells. Finally, we present functional data that strongly support the notion that our peptides affect integrin function in a selective, specific and dose-dependent fashion.

Phage display library screenings produce peptides that interact selectively with integrin cytoplasmic domains

We have successfully panned phage display peptide libraries to identify peptides that interact with the cytoplasmic domain of ß3 and/or ß5 subunits.

Panning of phage peptide libraries on ß3 or ß5 cytoplasmic domains.

We have isolated ß3 and ß5 cytoplasmic domain-binding peptides, by screening multiple phage libraries with recombinant GST fusion proteins that contain either GST-β3cyto or GST-ß5cyto coated onto microtiter wells. Immobilized GST was used as a negative control for enrichment during the panning on each cytoplasmic domain. Phage were sequenced from randomly selected clones after three rounds of panning as described elsewhere (43-45). We successfully isolated distinct sequences that interact specifically with the ß3 or with the ß5 cytoplasmic domains (Tables 1 and 2). Randomly selected clones from rounds II and III were sequenced. Amino acid sequences of the phagemid-encoded peptides were deduced from nucleotide sequences. The most frequent motifs found after panning with the indicated libraries are shown. The ratios were calculated by dividing the number of colonies recovered from β3-GST-coated wells and those recovered from GST or BSA.

Table 1. Sequences displayed by phage binding to ß3 integrin cytoplasmic domain; Table 2 Sequences displayed by phage binding to ß5 integrin cytoplasmic domain.

Table 1

PEPTIDE MOTIF	ß3/GST RATIO	ß3/BSA RATIO
CX_6C Library		
CEQRQTQEGC	4.3	14
CARLEVLLPC	2.8	18.7
X_4YX_4 Library		
YDWWYPWSW	5.6	163
GLDTYRGSP	4.1	48
SDNRYIGSW	3.3	32
YEWWYWSWA	2.2	281
KVSWYLDNG	2.1	20
SDWYYPWSW	2.1	157
AGWLYMSWK	1.8	2.4
Pool Cyclic Libraries		
CFQNRC	3.1	16
CNLSSEQC	2.7	62
CLRQSYSYNC	2.4	3.2

Table 2

PEPTIDE MOTIF	ß5/GST RATIO	ß5/BSA RATIO
Pool Cyclic libraries		
CYIWPDSGLC	5.2	193
CEPYWDGWFC	3.1	400
CKEDGWLMTC	2.3	836
CKLWQEDGY	1.8	665
CWDQNYLDDC	1.5	100
X_4YX_4 Library		
DEEGYYMMR	11.5	29
KQFSYRYLL	4.5	8
VVISYSMPD	3.8	28
SDWYYPWSW	2.4	304
DWFSYYEL	1.7	153

selected on GST-ß5cyto revealed several peptide motifs, as indicated in Table 3. Ratios were calculated as described.

The specificity of the interaction with ß3 or ß5 cytoplasmic domains was determined by calculating the ratios by using the number of phage bound to the cytoplasmic domain containing-fusion proteins (ß3 or ß5) and GST alone (the negative control). Figure 1

shows the results from binding assays performed with the GST-ß3cyto binding phage. We have tested six phage that display the motifs more frequently found during the second and third rounds of panning. Each panel shows the results from binding assays for the phage displaying different peptides that bind to the ß3 cytoplasmic domain, as indicated. Insertless phage or unselected libraries were used as negative controls and do not show binding above background. Two plating dilutions are shown for each assay.

A similar strategy was used to determine the specificity of each phage isolated in the screenings involving the ß5 cytoplasmic domain fusion protein. The binding assays were performed with individually amplified phage. Figure 2 shows the results from binding assays performed with ß5 cyto binding phage. We have tested four phage displaying the motifs that were found more frequently during the second and third rounds of panning. Each panel shows the binding assays for the phage displaying peptides that bind to the ß5 cytoplasmic domain. Insertless phage or unselected libraries were used as negative controls and do not show binding above background in these assays.

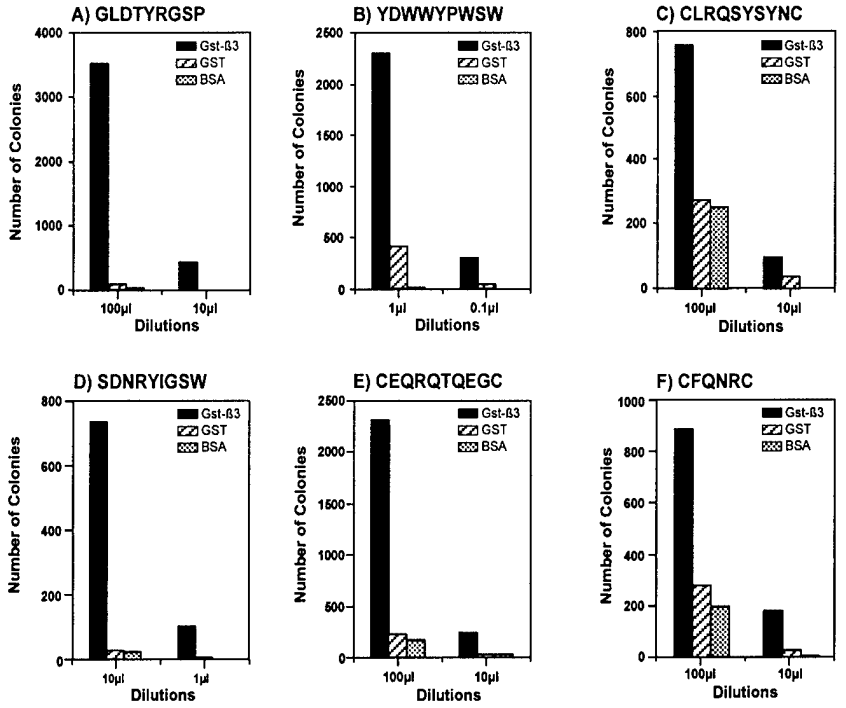


Figure 1. Binding of ß3 cytoplasmic domain-selected phage to immobilized proteins. GST fusion proteins or GST alone were coated on microtiter wells at 10 µg/ml and used to bind phage expressing the peptides shown inTable 2 (each phage is identified by the peptide sequence it displays, i.e. GLDTYRGSP; YDWWYPWSW; CLRQSYSNC; SDNRYIGSW; CEQRQTQEGC; CFQNRC). The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

To determine whether the binding of the selected motifs was specific for each cytoplasmic domain, we performed binding assays comparing the interaction of individual phage motifs with ß1, ß3, or ß5 cytoplasmic domain fusion proteins (Table 3). We have determined by ELISA with anti-GST antibodies that the three proteins can be coated onto plastic at equivalent efficiency, and thus that the differences in binding do not reflect differences in coating concentrations. As shown in Figures 3A and 3B, both the ß3- and ß5-selected phage interact preferably with the proteins on which they were originally selected. None of the phage tested seem to bind strongly to the ß1 cytoplasmic domain.

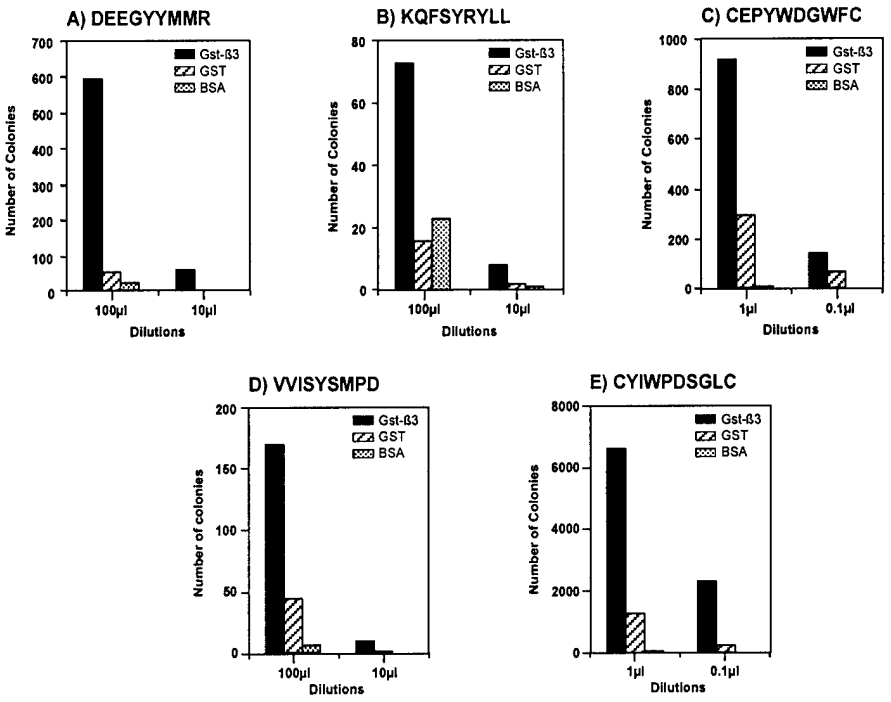


Figure 2. Binding of ß5 cytoplasmic domain-selected phage to immobilized proteins. GST fusion proteins or GST alone were coated on microtiter wells at 10 µg/ml and used to bind each phage expressing the peptides shown in Table 3 (each phage is identified by the peptide sequence it displays). (A) VVISYSMPD; (B) KQFSYRYLL; (C) CYIWPDSGLC; (D) CEPYWDGWFC (E) DEEGYYMMR. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

Table 3. Binding of ß3 and ß5 cytoplasmic domain-selected phage to immobilized proteins.

β3 MOTIF	β3/β1 RATIO	β3/β5 RATIO	β3/GST RATIO
CX_9 Library			
CEQRQTQEGC	**3.6**	**2.4**	**8.3**
CARLEVLLPC	**2.2**	**1.4**	**2.8**
X_4YX_4 Library			
SDNRYIGSW	**5.4**	**9.5**	**15.1**
GLDTYRGSP	**6.4**	**6.5**	**10.4**
YEWWYWSWA	**2.1**	**1.3**	**2.2**
YDWWYPWSW	**10.6**	**1.7**	**5.6**
SDWYYPWSW	**1.9**	**1.1**	**2.1**
Pool Cyclic Libraries			
CLRQSYSYNC	**1.1**	**1.5**	**2.4**
CFQNRC	**1.7**	**8**	**3.1**

ß5 MOTIF	β5/β1 RATIO	β5/β3 RATIO	β5/GST RATIO
Pool Cyclic Libraries			
CYIWPDSGLC	**7**	**12**	**3.2**
CEPYWDGWFC	**64**	**54**	**3.2**
X_4YX_4 Library			
DEEGYYMMR	**1.2**	**2.7**	**11.4**
DWFSYYEL	**1.7**	**1.5**	**1.2**
VVISYSMPD	**11.4**	**9.4**	**36.2**
KQFSYRYLL	**1.2**	**10.2**	**5.6**

Characterization of the synthetic peptides corresponding to the sequences displayed by the integrin-cytoplasmic domain-binding phage.

We selected specific phage for further studies on the basis of their binding properties. We used synthetic peptides corresponding to the sequence displayed by the phage to perform inhibitory studies. This assay is important because it determines whether phage binding is entirely mediated by the peptide displayed by the phage. As expected, we found that the synthetic peptides can inhibit the binding of the corresponding phage in a dose-dependent manner (Figures 4A and 4B). A control peptide containing unrelated amino acids had no effect on phage binding when tested at identical concentrations.

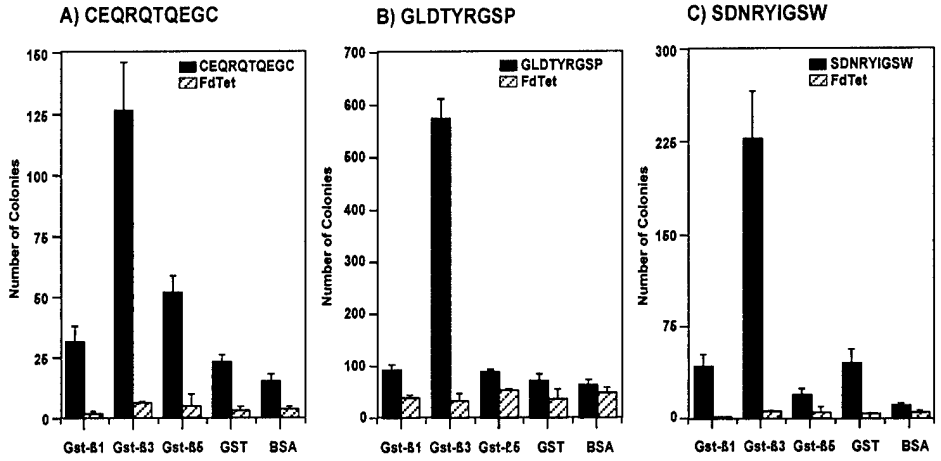


Figure 3A. Binding of ß3 cytoplasmic domain-selected phage to ß1, ß3 and ß5 immobilized proteins. GST fusion proteins or GST alone were coated on microtiter wells at 10 μg/ml and used to bind each phage expressing the peptides shown in Table 3 (each phage is identified by the peptide sequence it displays). The data represent the mean colony counts from triplicate wells with standard error less than 10% of the mean. Fd-tet insertless phage was used as a negative control.

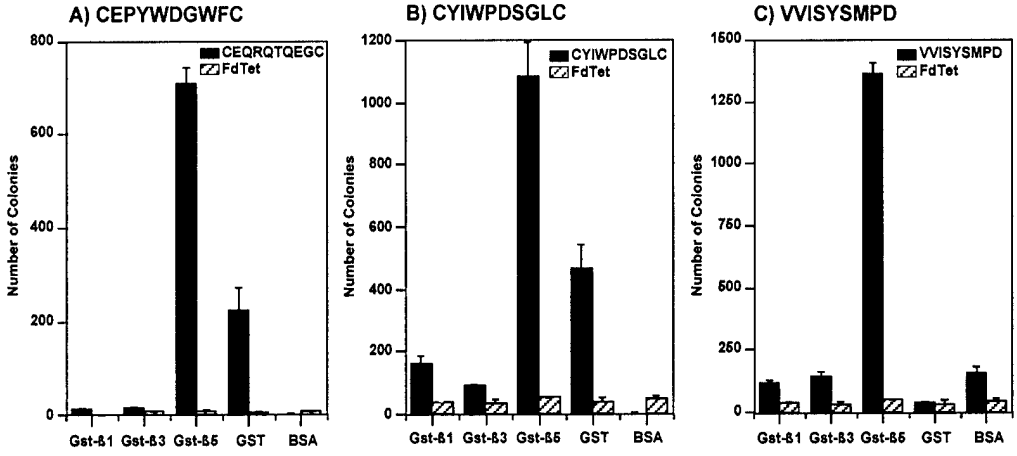


Figure 3B. Binding of ß5 cytoplasmic domain-selected phage to ß1, ß3 and ß5 immobilized proteins. GST fusion proteins or GST alone were coated on microtiter wells at 10 μg/ml and used to bind each phage expressing the peptides shown in Table 3 (each phage is identified by the peptide sequence it displays). The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean. Fd-tet insertless phage was used as a negative control.

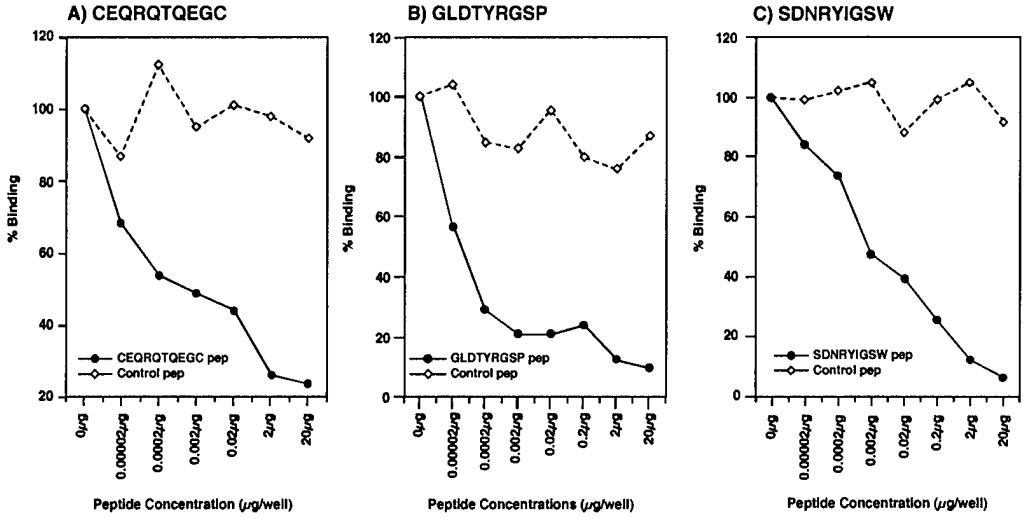


Figure 4A. Binding of the cytoplasmic-domain binding phage to ß3 immobilized protein and inhibition with the synthetic peptide. Phage were incubated on wells coated with GST-ß3cyto in the presence of increasing concentrations of the corresponding synthetic peptide or a control peptide. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

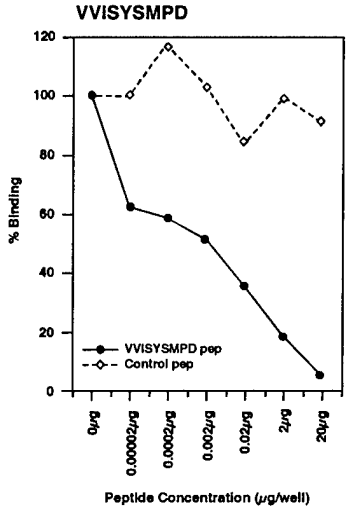


Figure 4B. Binding of the cytoplasmic-domain binding phage to ß5 immobilized protein and inhibition with the synthetic peptide. Phage were incubated on wells coated with GST-ß5cyto in the presence of increasing concentrations of the corresponding synthetic peptide or a control peptide. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

Phosphorylation events modulate the interaction of the selected peptides with cytoplasmic domains

Events involving phosphorylation are important in regulating signal transduction. We used the phage display system to evaluate the effect of tyrosine phosphorylation at two levels: (i) recombinant fusion proteins containing ß3 or ß5 cytoplasmic domains were used for panning of phage libraries displaying tyrosine-containing peptides or (ii) the cytoplasmic domains themselves were phosphorylated before phage selection was performed. Experiments were performed to investigate the capacity of specific tyrosine kinases to modulate the interaction of the selected peptides with the cytoplasmic

domains. This strategy is interesting because it can reveal the effect of phosphorylation on their binding properties at different levels.

The results obtained in the panning of phage libraries displaying tyrosine-containing peptides are shown in Table 4.

Table 4. Sequences displayed by phosphorylated phage binding to integrin cytoplasmic domains.

PEPTIDE MOTIF	Phosp/Unphosp	β3 or β5/GST	β3 or β5/BSA
β3 cytoplasmic domain			
GGGSYRHVE	13.2	1.5	5.3
RAILYRLAN	2.8	1.3	20
MLLGYRFEK	2.5	3.5	2.7
β5 cytoplasmic domain			
TMLRYTVRL	14.3	3.4	2.2
TMLRYFMFP	4.2	2.3	3.8
TLRKYFHSS	3.8	3.8	15.2
TLRKYFHSS	1.8	5.6	7.3

Randomly selected clones from rounds III and IV were sequenced from X4YX4 phosphorylated library with Fyn. Amino acid sequences of the phagemid-encoded peptides were deduced from nucleotide sequences. Table 5 shows the motifs found most frequently after the indicated libraries were panned with β3 or β5. The ratio of binding to β3 or β5 was calculated by dividing the number of β3 or β5 colonies by GST or BSA colonies found after panning. The ratio of binding to ß3 or ß5 with phosphorylated phage by Fyn versus unphosphorylated phage was calculated by dividing the number of colonies found after the panning.

We also have begun to evaluate the capacity of specific tyrosine kinases to phosphorylate isolated cytoplasmic domain-binding peptides. We investigated the effect of phosphorylation on the affinity and specificity of the cytoplasmic domain-binding. Phage displaying the ß3 and ß5 cytoplasmic domain-binding peptides were phosphorylated *in vitro* as described previously (56-58). We then investigated whether phosphorylation of the phage affected their interaction with the respective cytoplasmic domain in a phage-binding assay. For these experiments, phage were specifically phosphorylated *in vitro* by Fyn kinase. Specific phosphorylation of the tyrosine-containing peptide on the surface of the phage was confirmed by using ^{32}P-gamma dATP in the kinase reaction and by separating the phage pIII protein by SDS-PAGE.

After phosphorylating the phage *in vitro*, we found that their phosphorylation state increased the binding affinity and specificity to the ß3 integrin cytoplasmic domain (Figures 5A and 5B). The TLRKYFHSS phage was also tested in assays that included other GST-cytoplasmic domain fusion proteins to determine specificity (Figure 5C).

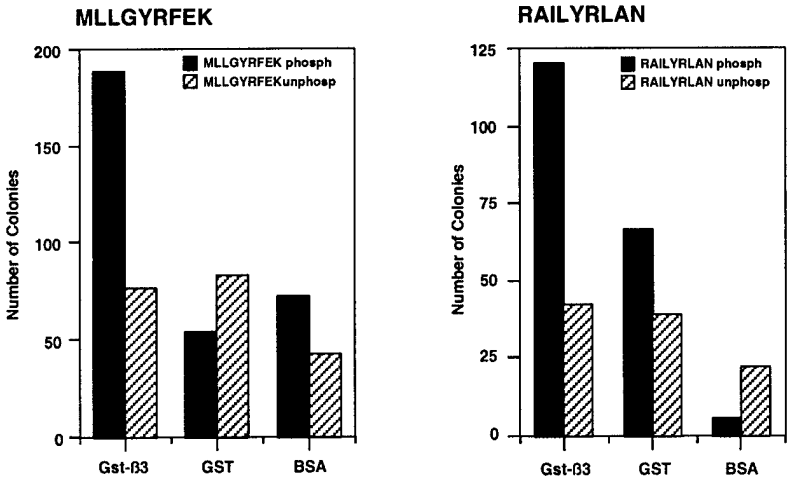


Figure 5A. Binding of phage to immobilized ß3-GST after phosphorylation. Phage were phosphorylated with Fyn kinase. Insertless phage were used as a control. Phage were incubated on wells coated with GST-ß3cyto. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

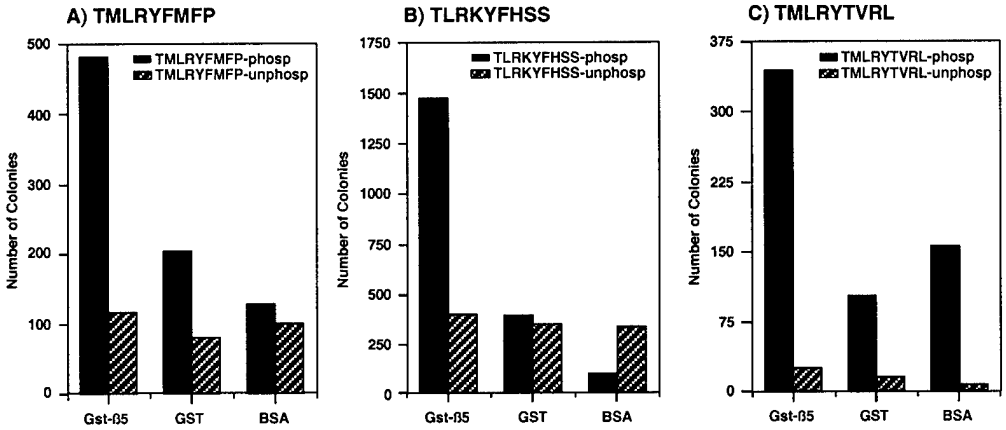


Figure 5B. Binding of phage to immobilized ß5-GST after phosphorylation. Phage were phosphorylated with Fyn kinase. Insertless phage were used as a control. Phage were incubated on wells coated with GST-ß3cyto. The data represent the mean of colony counts from triplicate wells, with standard error less than 10% of the mean.

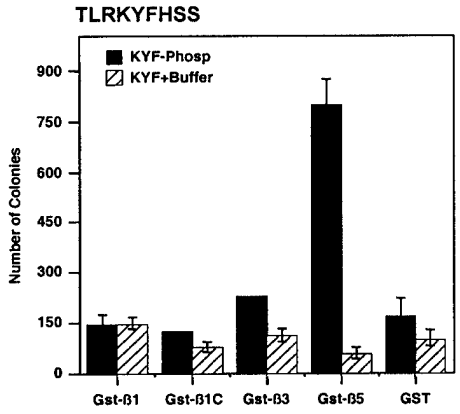
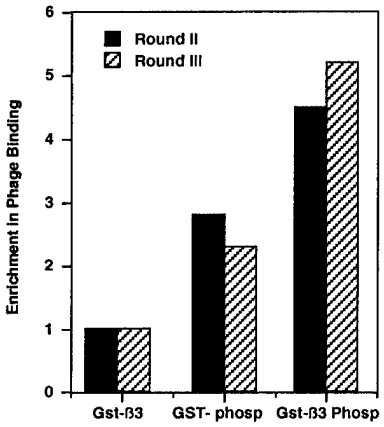


Figure 5C. Binding of phage to ß1, ß3 and ß5 immobilized GST fusion proteins after phosphorylation. Phages were phosphorylated with Fyn kinase. Insertless phage was used as a control. Phage were incubated on wells coated with GST-cytoplasmic domains. The data represent the mean of colony counts from triplicate wells, with standard error less than 10% of the mean.

Peptides that bind to the ß3 or ß5 cytoplasmic domains after phosphorylation in vitro are likely to be useful tools in understanding signal transduction regulation in the context of intact cells. We have performed screenings using that system. We have generated interesting data suggesting that it is possible to obtain peptides that will recognize the β3 cyto domain exclusively after phosphorylation (Figure 6).

Figure 6. Panning on phosphorylated GST-ß3 cyto. A GST fusion protein containing the ß3 cytoplasmic domain was immobilized onto microtiter wells at 10µg/ml before and after phosphorylation with Fyn kinase. The CX7C phage library was used. Bound phage were recovered after infection with K91 bacteria. Results illustrate the % of enrichment on phosphorylated GST-ß3 cyto versus that of phosphorylated GST after the second and third round of selection. The unphosphorylated GST-ß3 cyto was used as a control. The data represent the mean of duplicate wells, with standard error less than 10% of the mean.



Sequence similarity of integrin binding peptides with known cytoskeletal and signaling proteins.

Searching for sequence homology between these peptides and protein sequences found in the database, we found that the peptides displayed by integrin cytoplasmic domain-binding phage are similar to certain regions found within cytoskeletal proteins and proteins involved in signal transduction. At this point, despite the difficulty of evaluating what these similarities might mean, we plan to investigate whether they represent potentially relevant interactions. The similarity of some of the isolated peptides to a region of mitogen-activated protein kinase 5 (MAPK5, amino acids 227–234) is particularly interesting. Although the molecular mechanisms involved are not firmly identified as yet, a connection involving the MAPK cascade, cell adhesion, migration, and proliferation has been proposed (54). We plan to pursue studies with the most promising peptides

KEY RESEARCH ACCOMPLISHMENTS

- **We have selected peptides that bind specifically to ß3 or to ß5 integrin cytoplasmic domains.** We used use recombinant fusion proteins containing ß3 or ß5 cytoplasmic domains for biopanning of phage peptide libraries.

- **We have investigate whether phosphorylation events can modulate the interaction of the selected peptides with integrin cytoplasmic domains.** We used recombinant fusion proteins containing ß3 or ß5 cytoplasmic domains for biopanning of phage libraries displaying tyrosine-containing peptides. The libraries were phosphorylated *in vitro* using different kinases. We also studied the capacity of specific tyrosine kinases to phosphorylate isolated cytoplasmic domain binding peptides. The effect of phosphorylation on their binding properties were investigated.

- **We have determined some of the biological properties of the cytoplasmic domain-binding peptides.**

REPORTABLE OUTCOMES

A manuscript is in preparation to report the results of this project.

CONCLUSIONS

We have clearly demonstrated certain critical results that will contribute to the success of this project.

(i) evidence of successful introduction of peptides into cells and **(ii)** demonstration that the introduced peptides can affect cell function. Data addressing these issues are compiled in a manuscript in preparation. We also developed improved and more direct experimental strategies to complete the project.

Functional data are now available showing that the cytoplasmic domain-binding peptides selected on β3 or β5 can indeed interfere with integrin-mediated signaling and subsequent cellular responses (i.e., endothelial cell proliferation, and migration). We made a major investment to commercially obtain a panel of "internalizable" versions of our synthetic motifs found by phage screenings. These complex chimeric peptides consist of the most selective of the β3 or β5 cytoplasmic domain-binding peptides coupled to penetratin plus a biotin moiety to allow the peptides to be tracked once they are inside intact cells. Our results show that these membrane-permeable forms of the peptides (i) are indeed internalized (ii) may affect ß3 and ß5 post-ligand binding cellular events and (iii) can clearly induce massive apoptosis.

Identifying signaling events characteristic of tumor cells and angiogenic vasculature will improve our understanding of integrin biology and may suggest new therapeutic strategies for cancer and other diseases involving angiogenesis.

REFERENCES

1. Folkman, J. The role of angiogenesis in tumor growth. Semin. Cancer Biol. 3, 65-71 (1992).

2. Weinstat-Saslow, D. & Steeg, P.S. Angiogenesis and colonization in the tumor metastic process: basic and applied advances. FASED J. 8, 401-407 (1994).

3. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med. 1, 27-31 (1995).

4. Horak, E.R. et al. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicators of node metastases and survival in breast cancer. Lancet 340, 1120-1124 (1992).

5. Carrau, R.L. et al. Tumor angiogenesis as a predictor of tumor aggressiveness and metastatic potential in squamous cell carcinoma of the head and neck. Invasion Metastasis 15, 197-202 (1995).

6. Meitar, D., Crawford, S.E., Rademaker. A.W. & Cohn, S.L. Tumor angiogenesis correlates with metastatic disease, N-myc amplification, and poor outcome in human neuroblastoma. J. Clin. Oncol. 14, 405-414 (1996).

7. Takebayashi, Y., Aklyma,S., Yamada, K., S. & Aikou, T. Angiogenesis as an unfavorable prognostic factor in human colorectal carcinoma. Cancer 78, 226-231 (1996).

8. Hynes, R.O. Integrins: versatility, modulation and signaling in cell adhesion. Cell 69: 11-25 (1992).

9. Hemler M., Weitzman J., Pasqualini R., Kawaguchi S., Kassner P., and Berdichevsky F. Structure, biochemical properties, and biological functions of integrin cytoplasmic domains. In: Integrins: The Biological Problems (ed. Yoshi Takada) CRC Press, Inc., Boca Raton, FL, USA; pp.1-35. (1994).

10. Guan JL. Chen HC. Signal transduction in cell-matrix interactions. International Review of Cytology. 168:81-121, (1996).

11. LaFlamme, S.E., Auer, K.L. Integrin signaling. Seminars in Cancer Biology, 7(3): 111-8 (1996)

12. Ruoslahti E. RGD and other recognition sequences for integrins. Ann. Rev. Cell Dev. Biol. **12**:697-715, (1996).

13. Ruoslahti, E. Stretching is good for a cell. Science 276: 1345-1346, (1997).

14. Ruoslahti, E. Integrins as signaling molecules and targets for tumor therapy. Kidney International. 51(5): 1413-7, (1997).

15. LaFlamme, S.E., Akiyama, S.K., and Yamada, K.M. Regulation of fibronectin receptor distribution. J. Cell Biol. 117: 437-447 (1992).

16. Pasqualini, R., and Hemler, M.E. Contrasting roles for β1 and β5 cytoplasmic domains in subcellular localization, and cell migration. J. Cell Biol. 125: 447-60 (1994).

17. Lafrenie RM. Yamada KM. Integrin-dependent signal transduction Journal of Cellular Biochemistry. 61(4):543-53, (1996).

18. Shimizu Y. Intracellular signaling pathways and the regulation of cell adhesion. Human Cell. 9(3):175-80, (1996).

19. Shattil S.J., Ginsberg M.H. Perspectives series: cell adhesion in vascular biology. Integrin signaling in vascular biology. Journal of Clinical Investigation. 100(1): 1-5 (1997).

20. Drake, C.J., Cheresh, D.A., Little, C.D. An antagonist of αvβ3 prevents maturation of blood vessels during embryonic neovascularization. J. of Cell Science. 108: 2655-61, (1995).

21. Clark, R.A., Tonnesen, M.G., Gailit, J., Cheresh, D.A. Transient functional expression of αvβ3 on vascular cells during wound repair. American Journal of Pathology. 148(5):1407-21, (1996).

22. Brooks PC, Clark RA, and Cheresh DA. Requirement of vascular integrin αvß3 for angiogenesis. Science **264**:569-571, (1994).

23. Brooks PC, Montgomery AM, Rosenfeld M, Reinsfeld RA, Hu T, Klier G, and Cheresh DA. Integrin αvß3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell **79**: 1157-1164, (1994).

24. Brooks PC, Stromblad S, Klemle R, Visscher D, Sarkar FH, and Cheresh DA. Anti-integrin αvß3 blocks human breast cancer growth and angiogenesis in human skin. J. Clin. Invest. **96**:1815-1822, (1995).

25. Brooks PC. Klemke RL. Schon S. Lewis JM. Schwartz MA Insulin-like growth factor receptor cooperates with integrin αvß5 to promote tumor cell dissemination in vivo. Journal of Clinical Investigation. 99(6):1390-8, (1997).

26. Hammes H-P, Brownlee M, Joonczyk A, Sutter A, and Preissner KT. Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. Nature. Med. **5**:529-533, (1996).

27. Klemke, R.L., Yebra, M., Bayna, E.M., Cheresh, D.A. receptor tyrosine kinase signaling required for integrin αvß5-directed cell motility but not adhesion on vitronectin. J. of Cell Biology. 127(3): 859-66 (1994).

28. Lewis JM. Cheresh DA. Schwartz MA. Protein kinase C regulates αvß5-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. Journal of Cell Biology. 134(5):1323-32, (1996).

29. Friedlander M, Brooks PC, Sharffer RW, Kincaid CM, Varner JA, and Cheresh DA. Definition of two angiogenic pathways by distinct αv integrins. Science **270**: 1500-1502, (1995).

30. Varner JA. Brooks PC. Cheresh DA. REVIEW: the integrin αvß3: angiogenesis and apoptosis. Cell Adhesion & Communication. 3(4):367-74, (1995).

31. Varner JA. Cheresh DA. Integrins and cancer. Current Opinion in Cell Biology. 8(5):724-30, (1996).

32. Varner JA. Cheresh DA. Tumor angiogenesis and the role of vascular cell integrin αvß3. Important Advances in Oncology.: 69-87, (1996).

33. Filardo EJ. Cheresh DA. A structural basis for bi-directional integrin signaling. Princess Takamatsu Symposia. 24:106-17, (1994).

34. Shattil, S.J., O'Toole, T., Eigenthaler, M., Thon, V., Williams, M., Babior, B.M., and Gingsberg, M.H. ß3-endonexin, a novel polypeptide that interacts specifically with the cytoplasmic tail of the integrin ß3 subunit. J. Cell Biol. 131: 807-816, (1995).

35. Kashiwagi H. Schwartz MA. Eigenthaler M. Davis KA. Ginsberg MH. Shattil SJ. Affinity modulation of platelet integrin αIIbß3 by ß3-endonexin, a selective binding partner of the ß3 integrin cytoplasmic tail. Journal of Cell Biology. 137(6):1433-43, (1997).

36. Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C., and Burridge, K. Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. Nature 320: 531-533. (1986).

37. Pavalko, F.M., Otey, C.A., and Burridge, K. Identification of a filamin isoform enriched at the ends of stress fibers in chicken embryo fibroblasts. J. Cell Sci. 94: 109-118. (1989).

38. Otey, C.A., Pavalko, F.M., and Burridge, K. An interaction between a-actinin and the b 1 integrin subunit in vitro. J. Cell. Biol. 111: 721-729. (1990).

39. Otey, C.A., Vasquez, G.B., Burridge, K., and Erickson, B.W. Mapping of the a-actinin binding site within the ß1 integrin cytoplasmic domain. J. Biol. Chem. 268: 21193-21197. (1993).

40. Schaller, M.D., Otey, C.A., Hildebrand, J.D., and Parsons, J.T. Focal adhesion kinase and paxillin bind to peptides mimicking ß integrin cytoplasmic domains. J. Cell Biol. 130: 1181-1187. (1995).

41. Hannigan, G.E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M.G., Radeva, G., Filmus, J., Bell, J.C., and Dedhar, S. Regulation of cell adhesion and anchorage-dependent growth by a new ß1-integrin-linked protein kinase. Nature 379: 91-96. (1996).

42. Reddy, K.B., Gascard, P., Price, M.G., and Fox, JE. Identification and characterization of a specific interaction between skelemin and ß integrin cytoplasmic tails. Circulation 94:I-98a, (1996).

43. Koivunen E, Wang B, Dickinson CD, and Ruoslahti E. Peptides in cell adhesion research. Meth. Enzymol. **245**:346-369, (1994).

44. Koivunen E, Wang B, and Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. BioTechnol. **13**:265-270, (1995).

45. Pasqualini R, Koivunen E, and Ruoslahti E. A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. J. Cell Biol. **130**:1189-1196, (1995).

46. Robinson, M.J. & Cobb, M.H. Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol., 9: 180-186 (1997)

47. Seger, R. & Krebs, E.G. The MAPK signaling cascade. FASEB J., 9: 726-735 (1995).

48. Treisman, R. Regulation of transcription by MAP Kinase cascades. Curr Opin. Cell Biol., 8: 205-215 (1996).

50. Pikkarainen, T., Fornaro M., Feramisco J., Languino L., Pasqualini R., Ruoslahti E. ß1 integrin cytoplasmic domain peptide interacts with filamin and cyclin D. in preparation.

51. Koivunen E, Gay DA, Ruoslahti E. Selection of peptides binding to the α5β1 integrin from phage display library. J. Biol. Chem. **268**:20205-20210, (1993).

52. Smith GP, and Scott JK. Libraries of peptides and proteins displayed in filamentous phage. Meth. Enzymol. **21**:228-257, (1993).

53. Jenkins, A.L. et al. Tyrosine phosphorylation of the ß3 cytoplasmatic domain mediates integrin-cytoskeletal interactions. J. Biol. Chem 273(22): 13878-13885 (1998).

54. Lin, T.H., Aplin, A.E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., Juliano, R.L. Integrin-mediated activation of MAP kinase is dependent of FAK: evidence for dual integrin signaling pathways in fibroblast. J. Biol Chem. 136(6): 1385-1395 (1997).

55. Akiyama, S.K. Integrins in cell adhesion and signaling. Hum Cell. 9(3): 181-186 (1996).

56. Gram, H., Schmitz, R., Zuber,J.F., Baumann, G. Identification of phosphopeptide ligands for the Src-homology 2 (SH2) domain of Grb2 by phage display. Eur. J. Biochem. 246, 633-637, (1997).

57. Dente, L., Vetriani, C., Zucconi, A., Pelicci, G., Lanfrancone, L., Pelicci, P.G., Cesarini, G. Modified phage peptide libraries as a tool study specificity of phosphorylation and recognition of tyrosine containing peptides. J. Mol. Biol. 269, 294-703, (1997).

58. Schmitz, R., Baumann, G., Gram, H. Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src as assessed by phage display. J. Mol. Biol. 260, 664-677, (1996).

59. Herndier BG, Werner A, Arnstein P, Abbey NW, Demartis F, Cohen RL, Shuman MA and Levy, JA Characterization of a human Kaposi's sarcoma cell line that induces angiogenic tumors in animals. AIDS **8**:575-581, (1996).

60. Alberts, A.S., Thorburn, A.M., Shenolikar, S., Mumby, M.C. and Feramisco, J.R. Regulation of cell cycle progression and retinoblastoma protein function by protein phosphatases. Proc. Natl. Acad. Sci. USA. **90**:388-392, (1993).

61. Sosnowski, R.G., Feldman, S.T. and Feramisco, J.R. Cellular responses to wounding are blocked by interference of ras. J. Cell Biol.**121**:113-119, (1993).

62. La Morte, V., Kennedy, E., Collins, L., Harootunian, A., Brown, J. and Feramisco, J. A requirement for Ras protein in thrombin-stimulated mitogenesis in astrocytoma. J. Biol. Chem. **268**:19411-19415, (1993).

63. Feldman, S.T., Gjerset, R., Gately, D., Chien, K.R. and Feramisco, J.R. Expression of SV40 virus large T antigen by recombinant adenoviruses activates proliferation of corneal endothelium in vitro. J. Clin. Invest. **91**:1713-1720, (1993).

64. Al-Alawi, N., Rose, D.W., Mansour, S.J., Buckmaster, C., Ahn, N., Rapp, U., Meinkoth, J. and Feramisco, J.R. Thyrotropin-induced mitogenesis is Ras-dependent but appears to bypass the Raf-dependent cytoplasmic kinase cascade. Mol. Cell. Biol. **15**:1162-1168, (1995).

65. Prigent, S.A., Nangene, M., Lin, H., Huvar, I., Boss, G.R., Feramisco, J.R., Cavenee, W.K. and Huang, H-J.S. Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated EGF receptor is mediated by the RAS-SHC-GRB2 pathway. J Biol Chem **271**:25639-25645, (1996).

66. Kolch, W., Philipp, A., Mischak, H., Dutil, E.M., Mullen, T.-M., Feramisco, J.R., Meinkoth, J.L., and Rose, D.W. Inhibition of RAF-1 signaling by a monoclonal antibody, which interferes with RAF-1 activation and with MEK substrate binding. Oncogene **13:**1305-1314, (1996).

APPENDICES

N/A